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Ribosomes of Acid-Fast Bacilli: Immunogenicity, Serology, and In Vitro Correlates of Delayed Hypersensitivity

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Ribosomal fractions obtained from *Mycobacterium bovis* (BCG) and *M. smegmatis* (strain *butyricum*) were studied to determine their antigenicity, their ability to stimulate the production of soluble mediators of delayed hypersensitivity (in vitro correlates) by sensitized peritoneal exudate cells, and the antigenic relations of ribosomal antigens of BCG to BCG protoplasm and H37Rv culture filtrates. The crude ribosomes and the 50-30*S* ribosomal subunit pool obtained from each of the organisms induced both delayed and immediate hypersensitivity when injected in incomplete Freund adjuvant into rabbits, and skin reactions could be elicited in sensitized rabbits with those antigens. The crude ribosomes and 50-30*S* ribosomal subunit pool of *M. smegmatis* stimulated lymphocytes of guinea pigs sensitized with viable organisms to produce macrophage migration inhibition factor. Comparable ribosomal fractions from BCG bacilli caused lymphocytes of guinea pigs sensitized with viable *M. bovis* (BCG) to produce skin reactive factor. Immunoelectrophoretic studies showed that H37Rv culture filtrate, protoplasm, crude ribosomes, and 50-30*S* ribosomal subunits of BCG contain multiple precipitinogens and that many of these were shared between the different antigen systems. Comparative electrophoresis revealed that BCG protoplasm and H37Rv culture filtrate shared a major portion of their components with each other and relatively few with ribosomal systems. The ribosomal systems shared the major portion of their components with each other and relatively few with the other antigen systems.

Many attempts have been directed toward obtaining an antigen capable of provoking specific delayed hypersensitivity (DH) reactions in hosts sensitized with acid-fast bacilli. These have employed methods for isolation and fractionation of materials derived directly from the bacilli or from the media in which they were grown. By utilizing such methods (3, 12, 15, 22), it has been demonstrated that whole cells, cell walls, protoplasm, old tuberculin, purified protein derivative (PPD), and polypeptides derived from *Mycobacterium* species provoke reactions in sensitized hosts, but that these agents have varying degrees of specificity. As previously noted (1, 16), ribosomal preparations of *M. bovis* strain BCG (BCG) and *M. smegmatis* have also been shown to be active agents for provoking DH reactions.

In this study, both standard and comparative immunoelectrophoresis (IEP) have been used to compare the antigenic composition of various skin test antigens. No attempt has been made to ascertain all possible relationships between the ribosomal antigens and two reference antigen systems (i.e., protoplasm and an antigen system from culture filtrates of *M. tuberculosis* H37Rv). Studies of in vitro correlates of DH were done by using pools (50-30*S* pool) of BCG and *M. smegmatis* as antigens. The soluble mediators produced by stimulation of peritoneal exudate cells (PEC) with appropriate antigens were tested for the presence of migration inhibition factor (MIF) and skin reactive factor (SRF).

The results of the present study of in vitro correlates of DH conform to those previously obtained by in vivo tests (1) of the ability of mycobacterial ribosomes to provoke DH reactions in sensitized animals. They show that SRF and MIF are produced during cultivation of sensitized PEC with ribosomal preparations. Crude ribosomes (CR) and 50-30*S* ribosomal

subunit pools (50–30*S* pool) have been shown to be good immunogens for production of antibodies and DH in rabbits. The IEP studies establish the relationship of BCG ribosomal fractions to homologous protoplasm and to an H37Rv reference antigen system.

MATERIALS AND METHODS

Animals. Female Hartley strain guinea pigs and New Zealand white rabbits of either sex were obtained from local animal breeders.

Bacterial strains. The BCG strain of *M. bovis* originated at the Pasteur Institute, Paris, France. The Rocky Mountain Laboratory at Hamilton, Mont., supplied the culture of *M. smegmatis* (strain *butyricum*). The mycobacteria were maintained on Petragani medium or on Sauton potato slants.

Cultivation and storage of bacteria. BCG was grown on Sauton synthetic medium, whereas *M. smegmatis* was grown either on Sauton or on Lenert synthetic media (14). The bacteria were harvested and stored as previously described (1).

Antigen preparations. A reference antigen prepared from an unheated culture filtrate of H37Rv was obtained through the courtesy of G. S. Yee, Graphic Medicine Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md. The PPD-S was obtained from the Center for Disease Control, Tuberculosis Program, Atlanta, Ga. The method employed in the preparation of protoplasm, ultracentrifuged supernatant (UCS), and ribosomal fractions, as well as the chemical and physical analysis of these ribosomal preparations and ribosomal subunits, have previously been described (1).

Antisera preparation. The H37Rv reference antiserum was obtained from G. S. Yee. This antiserum was prepared in goats injected intramuscularly and subcutaneously (s.c.) with unheated H37Rv culture filtrate in complete Freund adjuvant (CFA). The BCG reference antiserum employed was prepared in our laboratory by s.c. injection of sheep with a mixture of whole organisms, cell walls, and protoplasm of BCG bacilli in CFA.

Separate groups of New Zealand rabbits were immunized for production of antiserum against CR, 50–30*S* pool, or UCS from both BCG and *M. smegmatis* by employing the methods of Estrup (6) and Freidman (7). The animals were injected in each hind footpad with 0.2 ml of incomplete Freund adjuvant (IFA) containing 1.5 mg of one of the antigens. Each group of rabbits received two booster doses of the same amount of a given antigen s.c. in IFA at 2-month intervals. Post-booster sera were obtained 7 days after the last injection, and the animals were tested for the presence of immediate and delayed sensitivity by intradermal (i.d.) injection of 0.2 ml of saline containing 10 μg of antigen. At the same time a similar amount of homologous protoplasm was injected at a different site. The sites of injection were measured and recorded as described below.

In vitro correlates of delayed hypersensitivity. The method of David et al. (5) was used for studies of MIF. Peritoneal exudates from normal guinea pigs and from guinea pigs sensitized with *M. smegmatis* were harvested 4 days after intraperitoneal injection of 30 ml of sterile mineral oil. The PEC were harvested, washed, drawn into capillary tubes, and centrifuged, and the tubes were cut at the cell-medium interface. These were then placed in sterile Sykes-Moore tissue culture chambers. Four to six capillary tubes contained in two to three Sykes-Moore tissue culture chambers were studied for each antigen. Cells were maintained at 37 C in chambers filled with Eagle minimum essential medium (MEM) containing 15% normal guinea pig serum. Experimental chambers also contained 15 μg per ml of CR or 50–30*S* pool from either BCG or *M. smegmatis*. Bacterial growth was inhibited by addition of 50 μg of streptomycin and 50 U of penicillin per ml. After incubation for 24 h, the migration pattern was recorded and the area of migration was determined by using a planimeter.

The method of Pick et al (20) was used for production of SRF. Briefly, oil-stimulated PEC from normal guinea pigs and guinea pigs sensitized with BCG were harvested as described above. Tissue cultures of these cells were maintained in MEM to which was added L-glutamine (100 mg/ml), sodium pyruvate (0.001 M), polyethylene glycol (20 M) (Carbowax-Dow Chemical Co.), penicillin (50 U/ml), streptomycin (50 μg/ml), and mycostatin (250 U/ml). Five milliliters of a cell suspension containing 1.2×10^7 cells per ml was incubated at 37 C for 24 h with 10 μg of either CR, 50–30*S* pool from strain BCG, or PPD-S per ml. Control flasks contained cells but no antigen. After incubation the suspensions were centrifuged at $100 \times g$ for 30 min to remove the cells, and the supernatant fluid was harvested. One set of control fluid was reconstituted with 10 μg of PPD-S per ml. Six normal guinea pigs and six guinea pigs sensitized with BCG were injected i.d. with 0.1 ml of each of these fluids. Two right-angle diameters of the lesions were measured at 3, 6, 12, and 24 h, and the thickness was determined by a Schnelltester. The volumes of the lesions were calculated (26), and only those having a volume of 15 mm^3 or greater (averaging 10 by 10 by 0.4 mm) were considered to be positive. The formula for this calculation is length times width times one-half thickness times 0.75.

Immunoelectrophoresis. Characterization and identification of BCG protoplasm, CR, and 50–30*S* pool, and the H37Rv reference antigen system were made by standard and comparative IEP employing three antisera. These were the H37Rv reference antiserum, BCG reference antiserum, and the rabbit BCG-CR antiserum. Standard IEP was performed by using the method of Scheidegger (21), and comparative IEP was performed by Osserman's technique (17). Barbitol buffer (pH 8.2, ionicity 0.1), 0.8% agarose, and 1:10,000 merthiolate were utilized in the IEP systems. Antigens were used at concentrations of 4 mg per ml and sera were used undiluted. The slides were subjected to electrophoresis in parallel at 5 V per cm.

The criteria used in evaluation of the results of IEP were: (i) enumeration of the number of arcs which developed after standard electrophoresis of a given antigen system, followed by reaction with the individ-

ual antibody systems; and (ii) determination of the number of precipitin arcs common to different antigen systems when developed with a given antibody system using comparative IEP. These criteria serve to establish, within the limits of the methods employed, the total number of immune precipitates in each antigen-antibody system and the number of immune precipitates in common among the various antigens.

RESULTS

Skin reactions in immunized rabbits. The hypersensitivity response of rabbits immunized with CR, 50–30*S* pool, or UCS derived from either BCG or *M. smegmatis* was tested. One week after administration of the second booster dose of antigen, skin tests were performed. The results of these tests are shown in Table 1. Ultracentrifuged supernatant fluid was found to be a poor sensitizing antigen, producing responsive states in only one of six animals. Crude ribosomes and 50–30*S* pool were effective sensitins. Either delayed, immediate, or mixed hypersensitivity reactions developed in 12 of 15 rabbits tested with these antigens; on the other hand, only 5 of these 15 animals responded to i.d. injection of homologous protoplasm.

Serological tests were performed by utilizing the sera of the above animals and the antigens with which they had been immunized. These were essentially ring tests performed with particulate ribosomal antigens and with soluble UCS antigens. All ribosomal antisera gave positive reactions. Only one of six animals immunized with UCS gave a minimal reaction with this antigen.

Macrophage (MIF). The results obtained from studies of migration of macrophages from oil-stimulated peritoneal exudates of normal and *M. smegmatis*-sensitized guinea pigs are summarized in Fig. 1 and Table 2. The PEC were cultured in the presence of CR or 50–30*S* pool from either BCG, *M. smegmatis*, or MEM alone. With the exception of CR from *M. smegmatis*, none of the antigens significantly decreased (Student's t test [$P = 0.05$]) the migration of macrophages of normal guinea pigs. The migration of macrophages in systems containing PEC of guinea pigs sensitized with *M. smegmatis* was definitely inhibited in the presence of the antigens utilized.

Skin reactive factor. The production of SRF by PEC of normal and BCG-sensitized guinea pigs exposed in vitro to 10 μg of either CR or 50–30*S* pool isolated from BCG bacilli or PPD-S are shown in Fig. 2 and 3. Undiluted supernatant fluids from normal PEC which had been incubated in vitro with any of the antigens usually failed to produce positive cutaneous reaction after injection into normal guinea pigs (Fig. 2A). Fluids from cells cultured with CR, however, produced moderate reactions 4 h after injection which were still present at 6 h but absent at 13 h.

Reactions were observed 6 h after injection of each of the above fluids into BCG-sensitized guinea pigs (Fig. 2B). The reactions caused by supernatant fluids from normal cells exposed to PPD-S or 50–30*S* pool were minimal at 12 and 24 h after injection, whereas those due to injection of supernatant fluid reconstituted with PPD-S or fluids from cells exposed to CR continued to increase in volume.

TABLE 1. *Results of skin tests of rabbits immunized with CR, 50–30S pool, and UCS from either BCG or M. smegmatis*[a]

Provoking antigen	Type of skin reaction	Reactions in animals immunized with:					
		BCG			*M. smegmatis*		
		UCS	CR	50–30*S* pool	UCS	CR	50–30*S* pool
Protoplasm[b]	Immediate[c]	0/3	0/6[d]	0/3	0/3	0/3	0/3
	Delayed[e]	0/3	2/6	1/3	1/3	1/3	1/3
	Total[f]	0/3	2/6	1/3	1/3	1/3	1/3
Immunizing antigen	Immediate	0/3	3/6	0/2	0/3	2/3	3/3
	Delayed	0/3	4/6	2/3	1/3	1/3	2/3
	Total	0/3	4/6	2/3	1/3	3/3	3/3

[a] Tested by i.d. injection of 10-μg amounts of the antigen used for immunization and homologous protoplasm at separate sites.
[b] Protoplasm from BCG or *M. smegmatis*, respectively.
[c] Readings made 7 h after injection of antigen.
[d] Total number of animals positive over the total number tested.
[e] Readings made 24 h after injection of antigen.
[f] Total number of animals exhibiting sensitivity over the total number tested.

Supernatant fluids from cultures of BCG-sensitized PEC exposed to each of the antigens provoked marked reactions in normal guinea pigs (Fig. 3A). Fluids from cells exposed to CR gave the most marked reactions. These reactions were present at 4 h, persisted for at least 13 h, and peaked at about 6 h. When the fluids were injected into BCG-sensitized guinea pigs (Fig. 3B), the resulting reactions persisted for at least 24 h, except for those due to injection of fluid from PEC incubated with PPD-S. It is of interest to note the biphasic nature and the intensity of the reactions which fluids from sensitized PEC incubated with CR produced after injection into BCG-sensitized animals.

Serological results. Comparisons were made of the H37Rv antigen, BCG protoplasm, BCG-CR, and BCG 50-30*S* pool, by using both standard and comparative IEP. It was reported by Janicki et al. (9) that standard IEP of the H37Rv antigen results in formation of two cathodal and seven anodal precipitin arcs 24 h after reaction with H37Rv antiserum, and that two additional precipitin arcs develop after 48 h. IEP studies in this investigation revealed that nine precipitin arcs developed by 24 h and that these were at the sites previously reported; however, the two precipitin arcs described as appearing after 48 h were not found in our patterns. Both standard and comparative IEP were performed with only one concentration of either antigen or antiserum. The results are shown in Tables 3, 4, and 5, and Fig. 4 and 5.

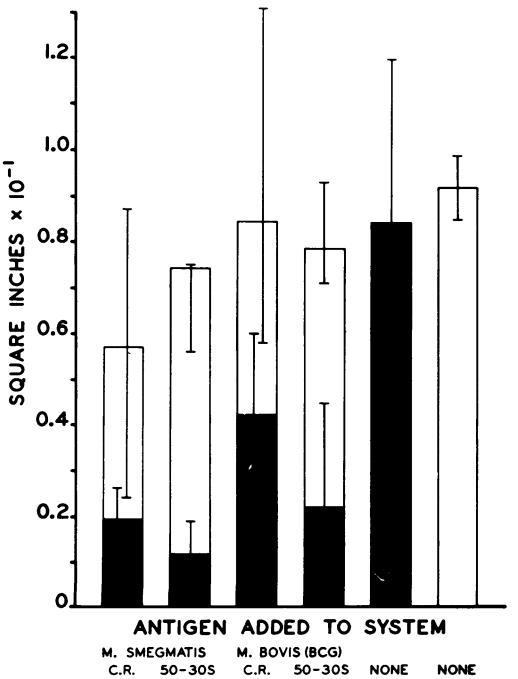

FIG. 1. *Results of macrophage migration inhibition tests. Mean area of spreading of macrophages of normal and M. smegmatis-sensitized guinea pigs exposed to 15 μg of either CR or 50-30S pool of either BCG or M. smegmatis. Range is indicated by brackets. Symbols: ■, M. smegmatis-sensitized PEC; □, normal PEC.*

TABLE 2. *Comparison of macrophage migration inhibition of normal and M. smegmatis-sensitized PEC cultured in the absence of antigen or the presence of either CR or 50-30S pool of either M. smegmatis or BCG*

Antigen added	Area of migration[a] of:		% Migration[b] of:	
	Normal cells	Sensitized cells	Normal cells	Sensitized cells
None	0.92	0.85		
M. smegmatis-CR	0.57	0.19	62	22
M. smegmatis-50-30*S*	0.74	0.12	81	14
BCG-CR	0.85	0.42	93	49
BCG-50-30*S* pool	0.78	0.22	85	26

[a] Area of migration in square inches $\times 10^{-1}$.

[b] Percent of migration equals area of migration of cells with antigen divided by area of migration of cells with no antigen times 100.

Standard IEP (Table 3) revealed that each of the antisera contained antibodies capable of reacting with some of the components of each antigen system. Antiserum produced against CR, however, gave relatively specific reactions, since 9 or 10 precipitin arcs developed after reaction with CR or 50-30*S* pool but only 4 developed after reaction with protoplasm or H37Rv reference antigen. The largest number of antigens detected in any antigen system varied from 9 to 11 and these were obtained by the use of specific antiserum.

Comparative IEP analysis (Table 4) yielded results of greater interest, since the number of antigens shared by each antigen system with H37Rv reference antigen could be determined. After electrophoresis of H37Rv reference antigen and subsequent addition of H37Rv immune serum to one trough and one of the other antigen systems (supplementary antigen) to the other, it was observed that BCG protoplasm shared the greater number of antigens with the H37Rv antigen system. BCG-whole cell antiserum gave similar results but revealed that only three precipitin arcs were shared by H37Rv reference antigen and BCG protoplasm and BCG-CR, and none were shared with 50-30*S* pool.

Figure 4 shows the maximum number of precipitin arcs developed by each of the antigen

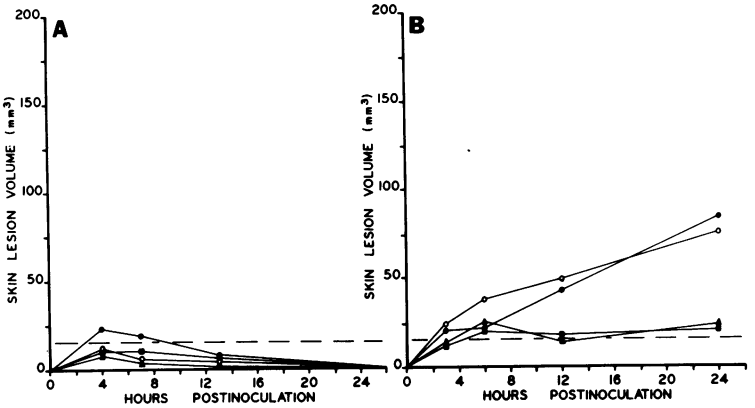

FIG. 2. *A, Skin reaction in normal guinea pig; B, skin reaction in BCG-sensitized guinea pig. SRF from normal PEC incubated in vitro with various antigens; 0.1 ml injected i.d. into normal and BCG-sensitized guinea pigs. Symbols: ○, control system reconstituted with 10 μg of PPD-S; ▲, SRF produced with 10 μg of PPD-S; ■, SRF produced with 10 μg of BCG-50-30S pool; ●, SRF produced with 10 μg of BCG-CR; --, positive reactions are 15 mm³ or greater.*

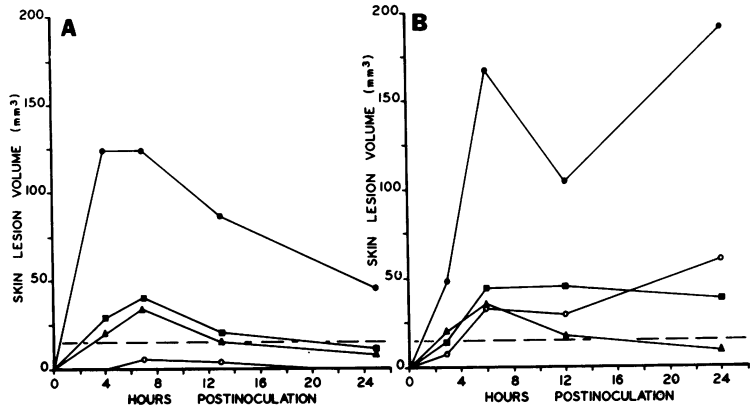

FIG. 3. *A, Skin reaction in normal guinea pig; B, skin reaction in BCG-sensitized guinea pig. SRF from BCG-sensitized PEC incubated in vitro with various antigens; 0.1 ml injected i.d. into normal and BCG-sensitized guinea pigs. Symbols: ○, control system reconstituted with 10 μg of PPD-S; ▲, SRF produced with 10 μg of PPD-S; ●, SRF produced with 10 μg of BCG-CR; ■, SRF produced with 10 μg of BCG-50-30S pool; --, positive reactions are 15 mm³ or greater.*

systems after reaction with each of the antisera after either standard (Table 3) or comparative (Table 4) IEP. Some idea of the specificity of the three antisera can be obtained if the number of these antibody components capable of reacting with the various antigen systems are compared. In this regard, H37Rv antiserum appears to have little specificity and cross-reacts freely with the three BCG antigens, whereas BCG-CR antiserum has specificity and separates the BCG ribosomal antigens from BCG protoplasm and H37Rv reference antigen.

The data in Table 5 show that, among the BCG antigen systems, the maximum number of antigens shared by CR and protoplasm or by 50-30S pool was nine. The maximum number of

TABLE 3. *Number of precipitin arcs developed by reaction of H37Rv reference antigen, BCG protoplasm, BCG-CR, and BCG-50-30S pool with antisera against H37Rv, BCG-whole cells, and BCG-CR*[a]

Antigen subjected to electrophoresis	No. of arcs registered with:		
	Anti-H37Rv	Anti-BCG-whole cell	Anti-BCG-CR
H37Rv reference antigen	9	7	4
BCG protoplasm	4	11	4
BCG-CR	7	10	9
BCG-50-30S pool	8	10	10

[a] Standard immunoelectrophoresis.

shared antigens found in each antigen system employing the identical sysystem as the supplementary antigen was 11 for protoplasm and 10 each for CR and 50-30*S* pool. The data in Table 4 show that the maximum number of antigens shared by H37Rv reference antigen is 8, 5, and 5 for BCG protoplasm, CR, and 50-30*S* pool, respectively. These relationships are depicted in Fig. 5 and indicate that the ribosomal antigens share few (5) antigens with the H37Rv antigen system, a greater number (7) with protoplasm, and the largest number (9) with each other.

Some of the individual IEP patterns analyzed above are shown in Fig. 6. Figure 6A shows the nine precipitin arcs developed after electrophoresis of H37Rv reference antigen and the four arcs developed by BCG protoplasm after reaction with H37Rv reference antiserum. Electrophoresis of the same antigens and development with anti-BCG-whole cell reference serum (Fig. 6B) resulted in production of 7 arcs with H37Rv reference antigen and 11 with BCG protoplasm. This same antiserum was used to obtain the results shown in Fig. 6C, in which CR was subjected to electrophoresis and 50-30*S* pool was included as the supplementary antigen. Nine identity reactions were observed between these two ribosomal antigens. In Fig. 6D, anti-CR serum was employed, and CR and a ribonucleic acid (RNA) preparation extracted from CR by the cold phenol method (23) were subjected to electrophoresis. The CR gave 10 immune precipitin arcs and the RNA preparation gave 2 arcs when developed with this antiserum.

TABLE 4. *Number of immune precipitin arcs in common among BCG protoplasm, BCG-CR, or BCG-50-30S pool and H37Rv antigen after electrophoresis of H37Rv antigen, and reaction with antisera against H37Rv, BCG-whole cell, and BCG-CR*[a]

Non-electrophoresed supplementary antigen	No. of H37Rv arcs[b] identifying with supplementary antigen		
	Anti-H37Rv	Anti-whole cell-BCG	Anti-BCG-CR
BCG protoplasm	8	7	3
BCG-CR	5	4	3
BCG-50-30*S* pool	3	5	0

[a] Comparative immunoelectrophoresis—Osserman technique.

[b] H37Rv antigen subjected to electrophoresis.

DISCUSSION

Although ribosomes from various mycobacteria (8, 25, 27, 28) have been isolated, it was only recently reported that ribosomes or ribosomal protein was tested for the ability to provoke delayed hypersensitivity reactions. Ribosomal protein from BCG (16) and ribosomes or ribosomal subunits from BCG and *M. smegmatis* have been shown to contain antigens capable of provoking delayed skin reactions in sensitized animals (1).

Results obtained from studies of rabbits immunized with CR, 50-30*S* pool, or UCS from either BCG or *M. smegmatis* indicate that UCS is a poor sensitin for induction of DH. Both CR and 50-30*S* pool, however, are good immunogens in this respect only when incorporated into IFA. The results obtained with UCS are similar to those described for protoplasm by Larson et al. (12), Beam (3), and Kanai and Youmans (11). Delayed reactions to protoplasm were observed in 5 of 15 (33%) rabbits immunized with CR or 50-30*S* pool (Table 1), whereas 9 of 15 (60%) of these animals exhibited DH reactions after i.d. injection of the specific antigen used for sensitization (Table 1). If the specific ribosomal antigen and protoplasm are compared on the basis of the total number of animals immunized which exhibit either delayed or immediate hypersensitivity, 5 of 15 (33%) of the rabbits react against protoplasm, whereas 12 of 15 (80%) react against ribosomal

TABLE 5. *Results of comparative immunoelectrophoresis tests (Osserman technique) showing the number of immune precipitin arcs in common among BCG protoplasm, BCG-CR and BCG-50-30S pool when tested by using antisera against H37Rv, BCG-whole cell, and BCG-CR*

Non-electrophoresed supplementary antigen	Antigen subjected to electrophoresis								
	BCG protoplasm			BCG-CR			BCG 50-30*S* ribosomal pool		
	Anti-H37Rv	Anti-BCG-whole cell	Anti-BCG-CR	Anti-H37Rv	Anti-BCG-whole cell	Anti-BCG-CR	Anti-H37Rv	Anti-BCG-whole cell	Anti-BCG-CR
BCG protoplasm	3	11	4	4	7	3	1	5	4
BCG-CR	4	6	3	4	10	5	1	6	9
BCG-50-30*S* pool	0	7	4	0	9	9	6	10	10

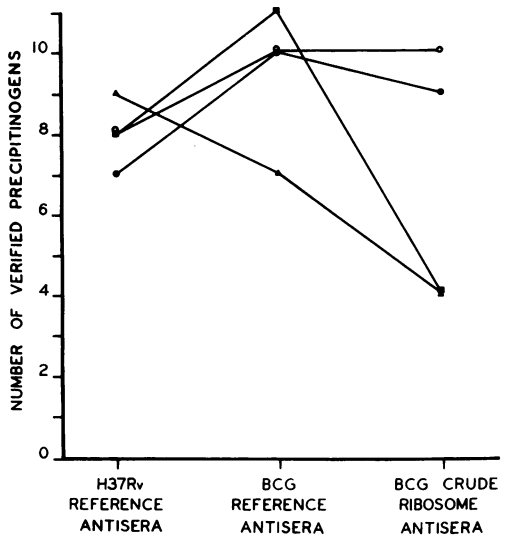

FIG. 4. *Number of precipitinogens observed when various ribosomal and reference antigens are subjected to electrophoresis and developed with various multispecific antisera. Symbols:* O, *BCG 50–30S pool;* ●, *BCG-CR;* ■, *BCG protoplasm;* ▲, *H37Rv reference antigen.*

antigens. Kanai et al. (11) found that two of five guinea pigs immunized with a particulate fraction of cells of *M. tuberculosis* (H37Ra) displayed DH to the same antigen. These animals developed lesions measuring 10 by 10 mm or more against this antigen but did not develop lesions after injection of old tuberculin. However, Youmans and Youmans (28) were unable to demonstrate that the ribosomal fractions of H37Ra induced DH in guinea pigs demonstrable by i.d. injection of PPD. These findings show that ribosomal antigens (including particulate fraction) are capable of inducing sensitivity in laboratory animals which may be detected by i.d. injections of ribosomal fractions but not by protoplasm or culture filtrate antigens.

Although BCG-UCS is a poor sensitin in rabbits, it will react in precipitin tests (unpublished data) with either BCG-whole cell or BCG-CR antiserum. It also cross-reacts with the ribosomal preparations in comparative IEP. As previously reported (1), BCG-UCS will also provoke DH in sensitized animals.

MIF and SRF are produced by PEC of guinea pigs sensitized with *M. smegmatis* and BCG, respectively, when such cells are exposed to ribosomal antigens. Migration of PEC of animals sensitized with viable *M. smegmatis* is markedly inhibited when these cells are incubated in the presence of either CR or 50–30*S* pool of strain BCG or *M. smegmatis*. In normal animals, *M. smegmatis*-CR was the only antigen found to cause a significant increase in MIF production when compared to controls.

Studies of production of SRF by PEC of normal guinea pigs show that incubation of normal cells with CR, 50–30*S* pool, or PPD-S does not result in the production of SRF. However, supernatant fluids from cells exposed to CR cause lesions when injected into the skin of guinea pigs sensitized by prior injection of viable BCG bacilli. These reactions appear to be due to antigens contained in the medium and develop slowly over the course of 24 h. Both PPD-S and 50–30*S* pool appear to be metabolized or altered during the incubation period in such a way that they are no longer effective skin test antigens, whereas CR is not so affected.

When supernatant fluids from BCG-sensitized cellls exposed to the above antigens are tested in normal guinea pigs, all preparations except for the reconstituted PPD control produce marked reactions 6 h after injection, and these decrease in size in 12 h. After injection of the supernatant fluid into sensitized guinea pigs, the early reaction to these fluids is accentuated, and there is either no change or an increase in the size of the lesions at 24 h. CR appears to be more effective in the production of SRF than 50–30*S* pool, but both ribosomal

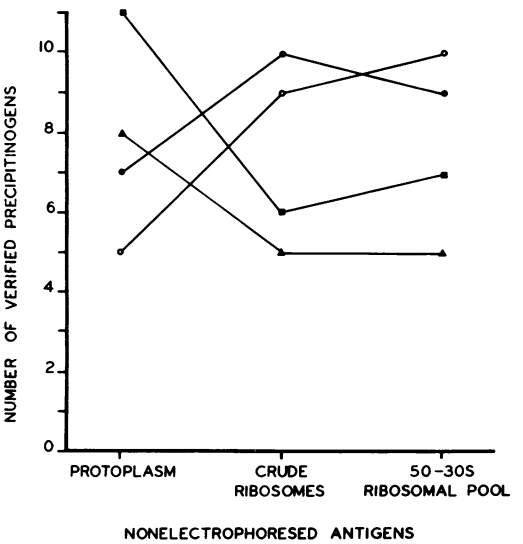

FIG. 5. *Maximum number of common antigens detected by comparative immunoelectrophoresis of BCG protoplasm, BCG-CR, BCG-50–30S pool, or H37Rv reference antigen when reacted with the three supplemental non-electrophoresed BCG antigen preparations in the presence of antisera raised against BCG-whole cells, BCG-CR, or H37Rv. Symbols:* O, *BCG-50–30S pool;* ●, *BCG-CR;* ■, *BCG protoplasm;* ▲, *H37Rv reference antigen.*

preparations appear to be effective stimulants for production and release of MIF.

The results of IEP analysis indicate that both of the BCG ribosomal preparations, BCG protoplasm and H37Rv antigen, contain essentially equivalent numbers of precipitinogens. The number of precipitin arcs developing after reaction of BCG protoplasm with BCG-whole cell antiserum is 11, of H37Rv reference antigen with H37Rv reference antiserum is 9, and of BCG-CR and 50–30*S* pool with BCG-CR antiserum is 10. The precipitinogens contained in each of these antigen systems are not identical, and differences are observed in the number of precipitinogens shared by them. BCG protoplasm contains eight antigens shared with H37Rv reference antigen, whereas BCG-CR and 50–30*S* pool share only five precipitinogens with the H37Rv antigen system. Results of comparative IEP indicate that there are two categories into which the different antigen systems may be included. The first category includes BCG protoplasm and H37Rv reference antigen. These react strongly with each other and weakly with the ribosomal antigen systems. The second includes both of the ribosomal antigens which again react strongly with themselves and less so with the other antigen systems.

This immunological analysis, together with results obtained from studies of the sensitivity and specificity of protoplasm, CR-50–30*S* pool, and 50*S* and 30*S* ribosomal subunits as agents for provoking delayed reactions in sensitized guinea pigs, suggest that the ribosomal antigens are different from protoplasm or culture filtrate antigens. The latter antigens are relatively nonspecific skin test agents and, as might be expected, share the major number of their components with each other. Since BCG and H37Rv are strains of closely related organisms, many of their proteins should be similar or identical. Both protoplasm and culture filtrates must contain a relatively unselected mixture of mycobacterial proteins, including some small amount of undegraded ribosomal elements. On the other hand, the ribosomes should contain a highly selected group of proteins associated with specific ribosomal RNA. Some of these, however, should show a greater or lesser degree of specificity for the organism from which they are isolated.

It has been demonstrated serologically with *Escherichia coli* that there is no extensive structural homology among the 55 proteins present in the ribosomes (6, 7, 10, 24). There is also evidence (13, 18) that in some cases, at least, strain specificity may be due to differences in the ribosomal protein complement (2, 4, 19). The results previously obtained from studies of ribosomal antigens as skin test agents show that 50*S* ribosomes are less specific antigens than 30*S* ribosomes, although both are potent in this respect. It was also found that the amount of 50*S* ribosomal subunits in the 50–30*S* pool is about two times that of the 30*S* ribosomal subunits. Since the IEP results were obtained with ribosomal antigens containing both 50*S* and 30*S* ribosomal subunits, the cross-reactions of CR and 50–30*S* pool with H37Rv culture filtrate antigen can be attributed to the presence of nonspecific antigens con-

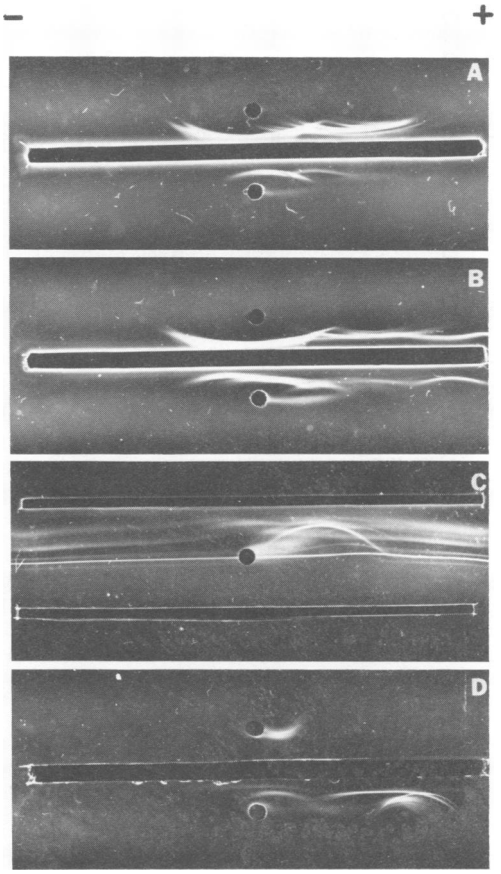

FIG. 6. *Immunoelectrophoretic patterns observed by using various ribosomal and reference antigens developed with multispecific antisera. Slide A, H37Rv reference antigen (top well) and BCG protoplasm (bottom well) were subjected to electrophoresis and developed with H37Rv reference antibody. Slide B, same antigens but developed with BCG reference antisera. Slide C, BCG-CR were subjected to electrophoresis and developed with BCG reference antisera (top trough). Supplemental, non-electrophoresed BCG-50–30S pool was placed in the bottom trough. Slide D, BCG-crude ribosomal RNA extract (top well) and BCG-50–30S pool (bottom well) were subject to electrophoresis with BCG-crude ribosomal antisera.*

tained in the 50S ribosomal subunits. Similarly, the sharing of almost all of the precipitinogens by CR and 50-30S pool can be attributed to the presence of proteins from both the 50S and 30S ribosomal subunits. These considerations are strengthened by recent findings (manuscript in preparation) that the core proteins of 30S ribosomal subunits of *M. smegmatis* are highly potent and specific skin test agents.

Immunoelectrophoretic analysis shows all the antigens under study to have similar total numbers of precipitinogens; however, the ribosomal preparations were found to have fewer cross-reactants to either protoplasm or H37Rv antigen. Antisera specificities were found to reside in the antisera prepared in rabbits against BCG-CR. Thus, it appears that much of the biological activity that is attributed to the protoplasm is also found in the ribosomal preparations.

Current work in progress is aimed at determining what proteins on the 30S ribosomal subunit are active in production of DH in properly sensitized animals and the biological properties of the ribosomal subunits in general.

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